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Use of PCR-Restriction Enzyme Pattern Analysis and Sequencing Database for *hsp65* Gene-Based Identification of *Nocardia* Species

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Nocardia **identification required laborious and time-consuming phenotypic and chemotaxonomic methods until molecular methods were developed in the mid-1990s. Here we reassessed the capacity of PCR-restriction enzyme pattern analysis (PRA) of the *hsp65* gene to differentiate *Nocardia* species, including 36 new species. Our results confirm that *hsp65* PRA must no longer be used for *Nocardia* species identification, as many species have the same restriction pattern. We then compared sequencing-based strategies using an *hsp65* database and a 16S rRNA database and found that the *hsp65* region contained sufficient polymorphisms for comprehensive *Nocardia* species identification.**

Nocardia species are gram-positive, weakly acid-fast, strictly aerobic bacteria that form filamentous branched cells which fragment into pleomorphic rod-shaped or coccoid elements. *Nocardia* species are essentially soil saprophytes involved in the decomposition of plant material (12, 16, 18). However, some species can infect both immunocompromised and immunocompetent individuals (18). Genus and species identification is necessary to predict antimicrobial susceptibility and for epidemiological purposes and also for environmental investigations (biodiversity, ecological niches, etc.).

Nocardia identification used to be based on laborious and time-consuming phenotypic and chemotaxonomic methods. Molecular methods were developed in the 1990s, including a 16S rRNA gene PCR-based method capable of distinguishing the genus *Nocardia* among aerobic actinomycetes (15). PCR-restriction enzyme pattern analysis (PRA) of a 441-bp fragment of the 65-kDa heat shock protein (*hsp65*) gene was developed to identify individual *Nocardia* species (28, 29). Sequential use of the two techniques provided rapid and simplified identification of *Nocardia* isolates from molecular dichotomous decision trees based on amplification/no amplification and the number and size of restriction fragments.

The genus *Nocardia* has undergone a taxonomic revolution during the last 10 years. Only 12 species were described between 1888, when the genus was first isolated by Nocard (20), and 1996, whereas more than 40 species are now recognized to exist. Some have been collected from clinical specimens; other have been isolated only from environmental specimens. PCR methods developed during the last decade have not yet been tested on the full range of known *Nocardia* species. For example, no data are available on the *hsp65*-PRA patterns of the 36 new species. Moreover, sequencing methods are increasingly important identification tools, and those based on 16S rRNA gene polymorphism have been applied to *Nocardia* (7, 19, 21). The MicroSeq 500 16S rRNA gene kit (PE Applied Biosystems) and the RIDOM database and BIBI database based on this methodology have recently been applied to the species identification of *Mycobacterium* and *Nocardia* isolates (6, 7, 9, 19, 21, 32). These approaches proved to be as efficient as conventional methods (biochemical tests, high-pressure liquid chromatography, and molecular probes) for many but not all *Nocardia* species (7). The latter authors underlined that public databases which are not monitored (no standard annotation, no control of strain identification, etc.) should be used with caution. Moreover, in order to overcome the strong similarity of 16S rRNA gene sequences within the genus *Mycobacterium* (e.g., *M. gastri* and *M. kansasii*) and microheterogeneity within a given species (e.g., *M. gordonae*), the study of other genes such as *hsp65* (23), *rpoB* (13), *sod* (35), *recA* (1), and 16S-23S *ITS* (24) may be used. Similar problems arise with *Nocardia* (19, 21).

Here we reevaluated the accuracy of the *hsp65*-PRA method and compared it with an alternative strategy based on partial *hsp65* gene sequences for *Nocardia* species identification.

MATERIALS AND METHODS

Type and reference strains. Forty-four strains corresponding to 44 species of *Nocardia* were studied (Table 1). *N. asteroides* ATCC 49872, representative of "*N. asteroides* type IV," was also included, as it corresponded to clearly individualized clusters (which have not yet been named) (4, 17). The strain ATCC 19247^T, previously used as a representative of *N. asteroides*, was included in this study. The taxonomic position of this strain has given rise to much controversy. This strain was in fact representative only of a rare unnamed subgroup of the former *N. asteroides* complex (21). In the same way, the strain ATCC 14759 was proposed as the reference strain for the type VI drug susceptibility pattern. But some authors indicated that *N. cyriacigeorgica* may be the same as the major group of isolates (i.e., type VI) within the *N. asteroides* complex (21, 25). In the absence of information (especially DNA-DNA homology and decision by taxonomic committees) (21) allowing a definitive conclusion, we decided to include the two species in our study and to present separately the data for the two

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TABLE 1. Strains of *Nocardia* studied

Nocardia species	Collection[a]	Reference	Origin	Yr of description	Accession no.	
					16S rRNA	*hsp65*
abscessus	DSM	44432^T	Human	2000	AY544980	AY544983
africana	DSM	44491^T	Human	2001	AY756540	AY756512
arthritidis	DSM	44731^T	Human	2004	AY903619	AY903633
araoensis	DSM	44729^T	Human	2004	AY903623	AY903637
asiatica	DSM	44668^T	Human	2004	AY903617	AY903631
asteroides	ATCC	19247^T	Human	1896	AY756541	AY756513
asteroides type IV	ATCC	49872	Human	1985	AY756542	AY756514
asteroides type VI	ATCC	14759	Human	1957	DQ223862	DQ223863
beijingensis	JCM	10666^T	Soil	2001	AY756543	AY756515
brasiliensis	ATCC	19296^T	Human	1913	AY756544	AY756516
brevicatena	DSM	43024^T	Human	1982	AY756545	AY756517
carnea	DSM	43397^T	Human	1913	AY756546	AY756518
cerradoensis	DSM	44546^T	Soil	2003	AY756547	AY756519
crassostreae	ATCC	700418^T	Oysters	1998	AY756548	AY756520
cummidelens	DSM	44490^T	Soil	2000	AY756549	AY756521
cyriacigeorgica	DSM	44484^T	Human	2001	AY756550	AY756522
farcinica	DSM	43665^T	Human	1889	AY756551	AY756523
flavorosea	JCM	3332^T	Soil	1998	AY756552	AY756524
fluminea	DSM	44489^T	Soil	2000	AY756553	AY756525
higoensis	DSM	44732^T	Human	2004	AY903620	AY903634
ignorata	DSM	44496^T	Human	2001	AY756554	AY756526
inohanensis	DSM	44667^T	Human	2004	AY903611	AY903625
mexicana	CIP	108295^T	Human	2004	AY903610	AY903624
neocaledoniensis	DSM	44717^T	Soil	2004	AY903614	AY903628
niigatensis	DSM	44670^T	Human	2004	AY903615	AY903629
nova	CIP	104777^T	Human	1983	AY756555	AY756527
otitidiscaviarum	ATCC	14629^T	Human	1924	AY756556	AY756528
paucivorans	DSM	44386^T	Human	2000	AY756557	AY756529
pneumoniae	DSM	44730^T	Human	2004	AY903622	AY903636
pseudobrasiliensis	DSM	44290^T	Human	1996	AY756558	AY756530
pseudovaccinii	DSM	43406^T	Plant	2002	AY756559	AY756531
puris	DSM	44599^T	Human	2003	AY903618	AY903632
salmonicida	JCM	4826^T	Fish	1999	AY756560	AY756532
seriolae	DSM	44129^T	Fish	1988	AY756561	AY756533
shimofusensis	DSM	44733^T	Soil	2004	AY903621	AY903635
soli	DSM	44488^T	Water	2000	AY756562	AY756534
tenerifensis	DSM	44704^T	Soil	2004	AY903613	AY903627
testacea	DSM	44765^T	Human	2004	AY903612	AY903626
transvalensis	DSM	43405^T	Human	1927	AY756563	AY756535
uniformis	JCM	3224^T	Soil	1999	AY756564	AY756536
vaccinii	ATCC	11092^T	Plant	1952	AY756565	AY756537
veterana	DSM	44445^T	Human	2001	AY756566	AY756538
vinacea	JCM	10988^T	Soil	2001	AY756567	AY756539
yamanashiensis	DSM	44669^T	Human	2004	AY903616	AY903630

[a] ATCC, American Type Culture Collection, Manassas, VA; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; JCM, Japan Collection of Microorganisms, Wako-Shi, Saitama, Japan; CIP, Collection Institut Pasteur, Paris, France.

representative strains. *Streptomyces somaliensis* DSM 41612^T was used as the outgroup for phylogenetic analysis. The strains were obtained from international collections and grown on Bennett agar at 37°C for 3 to 15 days.

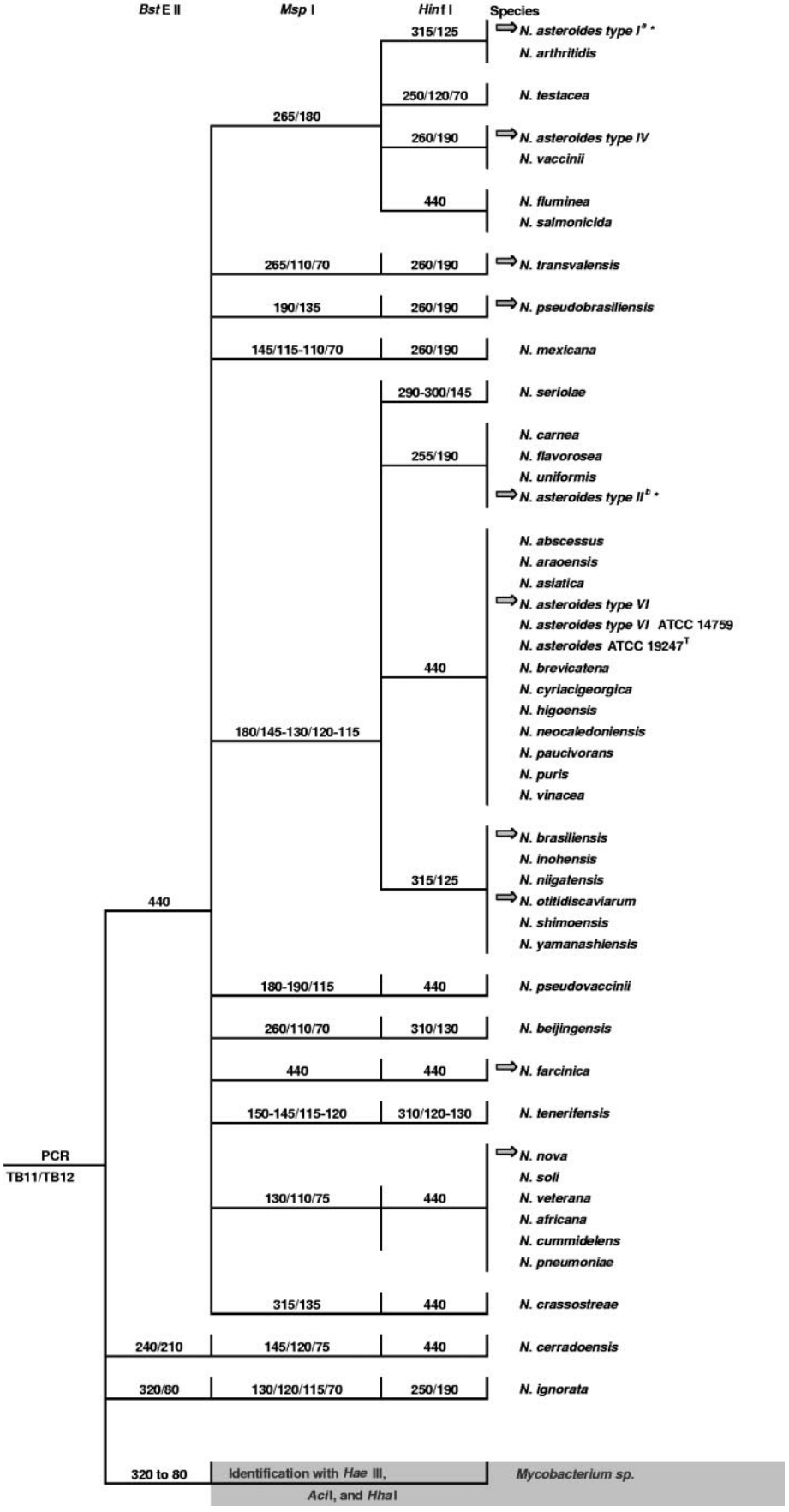
Clinical isolates. We also studied 21 clinical isolates sent for identification to the Observatoire Français des Nocardioses (Lyon, France). We confirmed that they belonged to the genus *Nocardia* by analyzing basic phenotypic characteristics such as culture morphology, mesodiaminopimelic acid, lysozyme resistance, substrate use (2), and also PCR (15).

DNA extraction. DNA was extracted with achromopeptidase. Colonies were picked off with a loop, and one loopful was suspended in 250 μl of sterile pyrolyzed water and vortexed for 1 minute. The bacterial filaments were crushed manually with conical plastic crushers. The mixture was then incubated for 15 min at 70°C. Fifty microliters of the suspension plus 1.5 μl of achromopeptidase (10 U/ml; Sigma, Steinheim, Germany) was incubated at 55°C for 15 min. The suspensions were then centrifuged for 3 min at 13,000 rpm. The supernatants were stored at −20°C until use.

***hsp65* amplification and PRA identification of *Nocardia* species.** A 441-bp fragment of the *hsp65* gene encoding the 65-kDa heat shock protein was amplified with primers described by Telenti et al. (TB11, 5′-ACCAACGATGGTGTG TCCAT-3′; TB12, 5′-CTTGTCGAACCGCATACCCT-3′) (30). Amplification was carried out in packaged PCR tubes (Ready-to-Go PCR Beads; Amersham Biosciences, Piscataway, N.J.) in a final volume of 25 μl (2.5 U of *Taq* polymerase puRe Taq, 10 mM Tris-HCl [pH 9], 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM each deoxynucleoside triphosphate) with 10 μl of DNA extract. Amplification was carried out in a thermal cycler (PTC-100; MJ Research, Boston, Mass.). Amplification runs included a 5-min initial denaturation step at 94°C, followed by 35 cycles (94°C for 60 s, 55°C for 60 s, and 72°C for 60 s), and a 10-min final extension step at 72°C.

Ten microliters of amplification product was digested with BstEII, MspI, and HinfI according to the manufacturer's instructions (New England Biolabs, Saint Quentin en Yvelines, France) and then electrophoresed in a 3% agarose gel (Agarose 3:1; Eurobio, Les Ulis, France) containing 0.5 μg/ml ethidium bromide (Sigma) at 90 V for 6 h. A 50-bp DNA ladder (Sigma) was used to interpret restriction patterns. The results were analyzed and included in a dichotomous decision tree based on the number and size of restriction fragments obtained with each enzyme, in the following order: BstEII, MspI, and HinfI.

***hsp65* and 16S rRNA gene sequencing and analysis.** A 606-bp fragment of the 16S rRNA gene was amplified with primers Noc1 (5′-GCTTAACACATGCAA


BstE II
Msp I
Hinf I
Species
PCR
TB11/TB12
315/125
N. asteroides type I[a] *
N. arthritidis
250/120/70
N. testacea
265/180
260/190
N. asteroides type IV
N. vaccinii
440
N. fluminea
N. salmonicida
265/110/70
260/190
N. transvalensis
190/135
260/190
N. pseudobrasiliensis
145/115-110/70
260/190
N. mexicana
290-300/145
N. seriolae
N. carnea
255/190
N. flavorosea
N. uniformis
N. asteroides type II[b] *
N. abscessus
N. araoensis
N. asiatica
N. asteroides type VI
N. asteroides type VI ATCC 14759
N. asteroides ATCC 19247[T]
440
N. brevicatena
N. cyriacigeorgica
N. higoensis
N. neocaledoniensis
180/145-130/120-115
N. paucivorans
N. puris
N. vinacea
N. brasiliensis
N. inohensis
315/125
N. nilgatensis
440
N. otitidiscaviarum
N. shimoensis
N. yamanashiensis
180-190/115
440
N. pseudovaccinii
260/110/70
310/130
N. beijingensis
440
440
N. farcinica
150-145/115-120
310/120-130
N. tenerifensis
N. nova
N. soli
130/110/75
440
N. veterana
N. africana
N. cummidelens
N. pneumoniae
315/135
440
N. crassostreae
240/210
145/120/75
440
N. cerradoensis
320/80
130/120/115/70
250/190
N. ignorata
320 to 80
Identification with Hae III,
AciI, and HhaI
Mycobacterium sp.

GTCG-3′) (positions 46 to 64, *Escherichia coli* numbering system) and Noc2 (5′-GAATTCCAGTCTCCCCTG-3′) (positions 663 to 680, *E. coli* numbering system). After 40 cycles of denaturation at 94°C for 60 s, primer annealing at 58°C for 60 s, and primer extension at 72°C for 60 s, followed by postamplification extension at 72°C for 5 min, PCR products were purified with the EZNA gel extraction kit (EAZY Nucleic Acid Purification; Omega Bio-Tek, Vaulx-en-Velin, France). For *hsp65* gene sequencing, the amplification protocol was the same as that used for *hsp65* PRA. Sequencing was performed with fluorescence-labeled dideoxynucleotide terminators implemented with the ABI PRISM Big Dye terminator cycle sequencing reaction kit (PE Applied Biosystems, Foster City, CA) and primers Noc1 and Noc2. The *hsp65* gene was amplified and sequenced with primers TB11 and TB12 as described below. Nucleotide sequences were determined with an ABI 377 automated sequencer according to the manufacturer's instructions (PE Applied Biosystems). Each sequence was manually aligned and analyzed to ensure a high quality of sequence data.

According to the 441-bp fragment of the *hsp65* gene, we achieved an in silico restriction analysis by using DNAStrider software. We tried to select new enzymes allowing a better differentiation of *Nocardia* species.

Moreover, the target 16S rRNA gene and *hsp65* nucleotide sequences were aligned with the Clustal W program (31). Phylo_win software (10) was used to infer evolutionary trees according to neighbor-joining methods (26) using the Kimura two-parameter model (14). Tree robustness was assessed by bootstrap resampling (1,000 replicates each).

Analysis of sequences from clinical strains. The *hsp65* and 16S rRNA sequences of the 21 clinical strains were determined as described above. They were then compared to the *Nocardia* entries in the *hsp65* and 16S rRNA databases that included only the sequences obtained in this study. The database comparison, using Bibi software (9), generated a list of the closest matches with pairwise distance scores indicating the percent difference between the unknown sequence and the database sequences.

RESULTS

Reference strains. Primers TB11 and TB12 amplified the expected 441-bp fragment of the *hsp65* gene for all the reference strains. The restriction patterns obtained for the species previously included in decision trees (Fig. 1, species indicated by arrows) conformed to those described by Steingrube et al. (28, 29). The restriction patterns of the 36 species not previously tested with this method (Fig. 1) were added to the previously published patterns. The new decision tree was much more complex. Thirteen species (*N. arthritidis*, *N. beijingensis*, *N. cerradoensis*, *N. crassostreae*, *N. farcinica*, *N. ignorata*, *N. mexicana*, *N. pseudobrasiliensis*, *N. pseudovaccinii*, *N. seriolae*, *N. tenerifensis*, *N. testacea*, and *N. transvalensis*) each had a unique restriction pattern. Conversely, a significant number of species had identical patterns that formed six clusters (Fig. 1), as follows: cluster 1, *N. asteroides* type IV (ATCC 49872) and *N. vaccinii*; cluster 2, *N. fluminea* and *N. salmonicida*; cluster 3, *N. carnea*, *N. flavorosea*, and *N. uniformis*; cluster 4, *N. abscessus*, *N. araoensis*, *N. asiatica*, *N. asteroides* type VI (ATCC 14759), *N. asteroides* ATCC 19247^T, *N. brevicatena*, *N. cyriacigeorgica*, *N. higoensis*, *N. neocaledoniensis*, *N. paucivorans*, *N. puris*, *N. vinacea*; cluster 5, *N. brasiliensis*, *N. inohanensis*, *N. niigatensis*, *N. otitidiscaviarum*, *N. shimofusensis*, and *N. yamanashiensis*; cluster 6, *N. africana*, *N. cummidelens*, *N. nova*, *N. pneumoniae*, *N. soli*, and *N. veterana*.

Surprisingly, the *N. cerradoensis* and *N. ignorata* amplification products had a BstEII restriction site, which is characteristic of mycobacteria (29).

According to these first results, we decided to generate a new decisional dichotomous tree with additional discriminating enzymes. More than 550 enzymes were tested in silico on the *hsp65* sequences of the 44 reference strains. Although some combinations of restriction enzymes provided better differentiation within a few clusters, none discriminated among all the species (data not shown). Importantly, the undifferentiated clusters included the species most commonly identified in clinical samples.

We then created two databases, one based on the *hsp65* gene sequence (401 bp) and the other based on the 16S rRNA gene sequence (569 bp) (excluding primer sequence regions), and compared their discriminatory power. Alignment of the *hsp65* sequences revealed a large number of variable nucleotide positions bearing at least one substitution in all species. The *hsp65* interspecies dissimilarity ranged from 12% between *N. fluminea* and *N. transvalensis* to 0% between *N. soli* and *N. cummidelens* and between *N. flavorosea* and *N. uniformis* (Table 2). By comparison, the interspecies dissimilarity based on the 16S rRNA fragment ranged from 9.5% between *N. fluminea* and *N. testacea* to 0% between *N. soli* and *N. cummidelens* and between *N. cyriacigeorgica* and *N. asteroides* type VI (Table 2). The number of variable sites was higher in the *hsp65* gene than in the 16S rRNA gene (26.4% versus 14.6%). Moreover, when we analyzed each pair of species individually (Table 2), 71% of pairwise distances (672/946) were higher with *hsp65* than with 16S rRNA.

Two phylogenetic trees were then generated from the *hsp65* and 16S rRNA gene sequences of the 44 species studied (Fig. 2 and 3). The *S. somaliensis* DSM 41612^T sequence was used to root both trees.

Clinical strains. Twenty-one isolates were submitted to 16S rRNA and *hsp65* sequence analysis using our own nocardial databases and Bibi software (http://pbil.univ-lyon1.fr/bibi/). For each clinical isolate and each fragment (16S rRNA and *hsp65*), we searched for the three closest species in the databases, e.g., the three species showing the lowest dissimilarity (Table 3). For 19 of the 21 isolates, the closest species were the same whether the *hsp65* or 16S rRNA sequences were used (Table 2). For the two remaining isolates (04.17 and 04.18), the *hsp65*-based analysis was inconclusive: the pairwise distances from *N. nova* and *N. africana* were equal, while those based on the 16S rRNA sequences were identical (isolate 04.18) or very close (isolate 04.17, 0.2% dissimilarity) to *N. nova*. In contrast, the two species were much more remote from *N. africana*, which was only the third most closely related species. The 16S rRNA sequence was thus more discriminatory.

For the other isolates, the *hsp65* gene was more discriminatory than the 16S rRNA gene. Indeed, the closest species were more distant (higher dissimilarity differences with *hsp65* sequences than with 16S rRNA sequences). For instance, the 16S rRNA sequence of isolate 04.07 showed 0.2% dissimilarity to *N. cerradoensis*, 0.3% dissimilarity to *N. veterana*, and 0.9% dissimilarity to *N. africana*. On the basis of the *hsp65* sequences, *N. cerradoensis* was the closest species (0.5% dissim-

FIG. 1. Steingrube's decision tree for the identification of *Nocardia* species by *hsp65* PRA (29), based on reference strains. The previously tested species are indicated by arrows. [a], reclassified as *N. abscessus* by Roth et al. (25); [b], reclassified as a *Nocardia* sp. by Roth et al. (25); *, results from the work of Steingrube et al. (29).

TABLE 2. 16S rRNA and *hsp65* gene sequence dissimilarities, with evolutionary distances, of 44 species of *Nocardia*, determined with ClustalW and Mega software

Strain no.[a]	Species	% Divergence in the 16S rRNA or *hsp65* gene of strain[b]:																																									
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44
1	*N. abscessus* DSM 44432[T]		4.6	2.8	2.3	0.2	2.5	3.5	2.6	2.3	2.6	4	4.4	4.2	5.6	6.2	2.6	2.3	4.9	6	2.3	5.8	5.3	6.5	2.8	6.2	4.8	5.3	4.2	3.5	6.3	6.5	3.3	5.6	6.7	2.3	6.2	3.7	5.8	3	4.9	5.1	4.6	5.6	5.3
2	*N. africana* DSM 44491[T]	4.5		4	5.1	4.8	4.2	2.5	3.5	5.1	5.1	3.5	5.5	1.2	3.2	6.2	3.5	4.6	5.3	5.6	6	6.3	5.1	4.9	4.8	4.8	1.8	2.5	2.8	4.8	4.4	4.2	4.2	6.5	4.9	6	6.2	6.5	6.5	3.2	4.2	2.3	0.7	3.7	4.6
3	*N. araoensis* DSM 44729[T]	1	4		1.2	3	4.6	4.4	4.7	1.6	4.6	2.8	6	4.2	5.6	6.2	4.8	4.4	6	6	4.9	5.8	6	4.6	5.3	5.6	3.9	4.8	2.8	1.1	5.6	5.5	4.9	6.2	5.5	4.9	6.2	3.9	7.2	4.4	6	4.4	4.4	4.4	5.1
4	*N. arthritidis* DSM 44731[T]	3.2	4.5	2.5		2.5	4.8	5.5	4.9	1.1	4.9	3.5	5.8	5.1	6.3	7.2	4.9	4.6	6.3	7	4.6	6.9	5.5	5.3	5.1	6.3	4.9	5.8	3.7	1.9	6.5	6.3	5.6	6.7	6.2	4.6	7.2	4.6	7.2	5.1	6.2	4.9	5.5	5.5	5.5
5	*N. asiatica* DSM 44668[T]	1.5	4	0.5	3		2.6	3.7	2.8	2.5	2.8	4.2	4.6	4.4	5.8	6.3	2.8	2.5	5.1	6.2	2.5	6	5.5	6.5	3	6.3	4.9	5.5	4.4	3.7	6.5	6.7	3.5	5.8	6.9	2.5	6.3	3.7	6	3.2	5.1	5.3	4.8	5.8	5.5
6	*N. asteroides* ATCC 19247[T]	4.2	6.5	3.2	5.2	2.7		4	3.9	4.4	3.5	5.3	5.1	4.4	5.6	4.9	3.9	3.5	5.8	5.5	2.5	4.4	6.3	7.4	1.4	6.3	4.4	5.8	5.3	4.6	5.5	4.6	4	5.1	6.5	2.5	4.9	5.1	5.8	4	5.3	4.6	4.4	3.9	5.3
7	*N. asteroides* type IV ATCC 49872[T]	5.5	5.7	5.5	5.7	5	5.5		3.2	5.8	4.2	4.9	5.6	3	4	5.5	3.2	3.5	5.1	4.9	5.1	5.6	6.2	4.9	4.8	5.8	3.5	3.9	4.2	5.1	5.6	5.5	4.2	6	6.2	5.1	5.5	5.5	6	0.9	5.3	4.8	2.5	4.9	5.8
8	*N. asteroides* type VI ATCC 14759	2.5	5.2	2	4	2	3.7	6		4.6	3	3.7	4	3.5	4	6.9	0	2.5	3.7	6.3	4.2	6.5	5.1	6.3	4.2	5.3	3.3	3.9	3.2	5.6	5.4	6.3	3	6.3	5.8	4.2	6.9	5.1	4.4	3	3.7	4.6	3.5	5.4	5.6
9	*N. beijingensis* JCM 10666[T]	4.2	5	3.5	1.7	3.7	6	7	5		3.9	3.5	5.6	5.1	5.3	6.2	4.6	4.6	6.2	6	4.6	5.8	5.5	4.2	4.8	5.6	3.9	5.5	3.7	2.3	5.5	5.6	5.6	5.6	5.8	4.6	6.2	3.7	7	5.3	5.1	4.9	5.5	4.8	5.5
10	*N. brasiliensis* ATCC 19296[T]	4.2	6.2	4.5	4.5	4.2	4	6.7	5	5		5.6	5.6	5.1	4.2	5.5	3	3	5.8	4.9	3.5	4.8	6	6	4	5.5	4.8	5.6	5.8	5.1	5.5	6	4.4	5.3	6.2	3.5	5.5	3.9	6.3	4.2	4.9	5.6	5.3	5.3	5.6
11	*N. brevicatena* DSM 43024[T]	4.5	5.2	3.5	5.5	3.5	5	7	4.5	6	6.2		4.2	3.3	4.9	8.1	3.7	5.8	3.7	7.7	6	7.6	6	5.6	5.6	5.6	4.2	4.6	1.1	2.8	5.6	5.8	2.8	8.1	5.3	6	8.1	6.3	4.9	4.8	4.9	3	3.9	4.8	5.3
12	*N. carnea* DSM 43397[T]	6	8.7	5.5	6.5	5.5	7.2	8.5	7	7.2	7.2	6		5.8	6.5	8.3	4	6.2	1.6	8.3	5.6	8.1	7.4	8.6	5.1	7.7	6.5	6.5	4.6	6.2	7.2	7.2	3.5	7.6	7	5.6	8.3	7.4	2.1	5.6	6.3	5.6	6.2	6.7	6.7
13	*N. cerradoensis* DSM 44546[T]	4.5	1	4	4.5	4	6.5	6.2	5.2	4.7	6.2	5.7	8.7		3.9	6.5	3.5	4.9	5.3	6	6.2	6.7	4.8	5.8	5.1	4.9	1.9	2.3	2.6	4.6	4.8	4.9	4	6.9	5.1	6.2	6.5	6.5	6.5	3.5	4	2.1	0.5	4.2	4.4
14	*N. crassostreae* ATCC 70418[T]	6.7	7.7	6	6.5	5.5	6.7	8.2	7	7.2	7.5	7.5	9.2	7.7		6.7	4	5.5	6	6.7	6.5	6.7	4.9	3.5	6.3	3.9	3.2	3.2	4.4	6	3.7	5.3	5.5	7.4	4.2	6.5	6.7	5.8	6.5	4.2	4.2	4.8	3.3	4.8	5.8
15	*N. cummidelens* DSM 44490[T]	6.7	8.5	6.7	5.7	7	6.5	10.2	8	5.5	5.7	7.7	8.5	8	10		6.9	6.9	9	1.4	6.7	0.9	6.7	6.7	5.8	6.7	4.9	7	7.4	6.5	6	5.8	7	1.1	7	6.7	0	5.1	9.3	5.8	6.3	6.7	6.3	5.6	5.8
16	*N. cyriacigeorgica* DSM 44484[T]	3	5.7	2.5	4.5	2.2	4	6.2	0.5	5	5	5	7.2	5.7	7.2	8		2.5	3.7	6.3	4.2	6.5	5.1	6.3	4.2	5.3	3.3	3.9	3.2	5.6	5.5	6.3	3	6.3	5.8	4.2	6.9	5.1	4.4	3	3.7	4.6	3.5	5.5	5.6
17	*N. farcinica* DSM 43665[T]	4	4.7	3.5	4.5	4	6.2	6.5	4.5	5.5	6.2	4.5	7.5	5.2	7	8.7	5		5.5	6.3	1.8	6.5	6.5	6.7	2.5	6.3	5.1	5.6	5.3	5.3	7.2	5.6	3.7	6.3	7	1.8	6.9	5.1	6.2	3.3	5.8	5.6	4.6	6.2	7
18	*N. flavorosea* JCM 3332[T]	6.2	9	5.7	7.5	5.7	7.5	8.7	6.7	8.2	8.5	6.5	1.5	9	9.5	9.5	7	7.7		7.9	6.2	8.1	7.9	8.1	5.5	7	5.6	6	4	6.3	7.7	7.4	3.3	9	7	6.2	9	7.2	1.6	5.1	6.2	5.6	5.6	7	7.6
19	*N. fluminea* DSM 44489[T]	7.2	9.7	7.2	6.2	7.5	6.7	10.5	8.2	6.5	6	8	8.7	9.2	10	1.2	8.2	9	9.7		7.2	1.1	7.2	6.5	6	6.2	4.6	6.7	7.4	6.5	6.9	6.7	7	1.9	6.5	7.2	1.4	4.9	9.5	5.6	6.3	6.9	5.8	6.3	6.3
20	*N. higoensis* DSM 44732[T]	2	5.2	2.5	4.5	2.5	4.7	6.5	2.5	4.7	4.7	5	6.5	5.2	7	7.2	2.7	5	6.7	7.5		6.3	6.7	7.9	1.9	7	6.7	7.2	6.2	5.1	7	5.5	3.3	6.9	7.6	0.4	6.7	5.8	6.3	4.8	6.7	6	6.2	6.2	6.3
21	*N. ignorata* DSM 44496[T]	6.5	8	6	6.7	6.2	6.2	10	7.7	6.2	6.7	7.5	7.7	7.5	10.2	2	7.7	8.2	8.7	3.2	7.5		6.9	6.9	5.3	6	4.8	7.2	7.2	6.2	6.2	6	6.5	1.6	6.7	6.3	0.9	4.8	8.8	5.8	6.5	6.5	6.5	5.5	6
22	*N. inohanensis* DSM 44667[T]	6.2	8.2	6.5	7.5	6.7	5.7	8.7	4.7	7	5.7	7.5	9	7.7	5.7	6.2	8	7.5	9.2	6.5	6.2	7		6.2	6.9	3	4.6	4	5.3	6	4.6	7.9	6.3	6.7	4.8	6.7	6.7	5.6	7.9	6	4.8	5.5	5.1	7.4	1.8
23	*N. mexicana* CIP 108295[T]	4.2	5.5	3.7	5.5	3.2	4.5	3	3.5	5.2	6.5	5.2	6.7	6	6.5	8.5	5	5.7	7	9.2	5.2	7.7	6.7		7.9	6.2	4.4	4.9	5.3	5.3	5.1	6.7	7.7	7.2	6.2	7.9	6.7	5.3	9.2	4.9	5.5	6	5.3	6.2	7.2
24	*N. neocaledoniensis* DSM 44717[T]	3.5	5.2	2.5	4.5	2	2.7	5.5	6.2	5.7	4.2	4.7	7	4.7	6	7.2	3.7	5	7.2	7.5	4	6.5	6.7	4.7		6.5	5.1	6.5	6	5.3	6.7	4.4	2.8	5.3	7	1.9	5.8	5.6	5.8	4.6	5.6	4.4	5.1	5.1	6.2
25	*N. niigatensis* DSM 44670[T]	5.5	7.5	5.7	6.2	5.2	5.2	7	5.2	7.5	5.7	7.2	9	7.5	4	9.2	6.5	6.7	9.2	9.2	5.2	10	5	6.7	4.7		3.3	3.7	5.5	5.6	3.5	6.5	5.6	7	4	7	6.7	5.1	7.6	6.2	5.5	4.9	4.8	6.5	3
26	*N. nova* CIP 104777[T]	4.5	0.5	4	4.5	4	6.5	5.7	4.5	5	6.2	5.2	8.7	1	7.7	8.5	5.7	4.7	9	9.7	5.2	8	8.2	5.5	5.2	7.5		2.8	3.2	4.4	3.2	4.4	4.9	5.5	5.3	6.7	4.9	4.9	6.9	4	3.7	3.3	1.8	3.9	4.4
27	*N. otitidiscaviarum* ATCC 14629[T]	3.5	5.7	3.2	4.2	3.2	5.5	6.2	4.7	5.7	6	4.2	7.2	5.7	6.2	8.7	5	5.2	7.5	9.2	5	8.2	7.2	5.5	4.7	5.5	5.7		3.5	5.5	3.5	5.8	5.6	7.6	4	7.2	7	5.1	7.2	4.6	3.5	4	2.1	5.3	4
28	*N. paucivorans* DSM 44386[T]	4.2	5.5	3.7	5.5	3.7	5.5	6.7	2	6.5	6.2	2.2	5.7	6	7	8.2	5.2	5.2	6.2	8.5	5.7	8	8	5	5.2	6.7	5.5	4.7		3	5.3	5.5	3.5	7.7	4.8	6.2	7.4	6.5	4.9	4.2	4.2	2.6	3.2	4.8	4.9
29	*N. pneumoniae* DSM 44730[T]	2.2	4.2	1.2	3.2	1.2	4	5.7	5.7	3.7	5	3.7	6.2	4.2	6.2	7	2.5	4.2	6.2	8	3.2	6.7	7.2	3.5	3.2	5.5	4.2	3.5	4		5.8	5.6	4.9	6.7	5.5	5.1	6.5	4.9	7.2	5.3	6.3	4.2	5.1	4.8	5.1
30	*N. pseudobrasiliensis* DSM 44290[T]	5.2	5.7	5.7	7.7	5.7	6	4.2	3.5	7.7	7.5	5.7	8.7	6.2	8.5	9.7	6.2	7	8.5	10	6.2	8.5	7	3.5	7.2	8.7	5.7	7	6	6.2		5.1	5.8	6.9	6.2	7	6	5.3	7.7	5.8	4.8	4.9	4.6	4.9	3.7
31	*N. pseudovaccinii* DSM 43406[T]	3.5	6.2	3	5.5	2.7	3.5	6.2	3.2	6	5	5.2	7.7	6.2	6.2	7.5	3.5	6	8	7.2	4.2	6.7	6.5	5	3.2	6.7	6.2	5.7	5.7	3.7	6.7		5.3	6.7	6.3	5.5	5.8	7	7	5.8	6	3.7	4.4	1.2	7
32	*N. puris* DSM 44599[T]	3.2	6	2.7	4.2	2.5	2.2	6.5	8	5	3.7	4.5	7	6	6.5	5.2	3.2	5.2	7.2	5.5	4	6	5.7	5.2	2.5	5.2	6	5	5	3.5	7	2.7		6.9	6.7	3.3	7	6.2	4.2	4	5.3	3.7	4.6	5.6	5.5
33	*N. salmonicida* JCM 4826[T]	7	9.5	7	6	7.2	6.5	10.2	6	6.2	5.7	7.7	8.5	9	9.7	1	8	8.7	9.5	0.2	7.2	3	6.2	9	7.2	9	9.5	9	8.2	7.7	9.7	7	5.2		7.4	6.9	1.1	4.9	9.2	6	6.5	6.7	6.9	6	6.2
34	*N. seriolae* DSM 44129[T]	5.2	7.7	5.5	7	5	5	7.2	1.5	8.2	6.2	7.5	9.2	7.7	4.2	10	6.2	7	9.5	10	5.5	9.7	5.7	7	5	1.2	7.7	5.2	7	5.7	8	7	5.5	9.7		7.6	7	7.2	7.6	6.9	6.2	4.8	5.1	6.3	4.4
35	*N. shimofusensis* DSM 44733[T]	2.5	5.7	2	4.5	2	4	6	8	5.2	5.7	5	6.5	5.7	7	8.2	1.7	4	6.7	8.5	2	7.5	7.5	4.2	3.5	6	5.7	5	5.2	2.7	6.2	3.7	4	8.2	5.7		6.7	5.8	6.3	4.8	6.7	6	6.2	6.2	6.3
36	*N. soli* DSM 44488[T]	6.7	8.5	6.7	5.7	7	6.5	10.2	3.7	5.5	5.7	7.7	8.5	8	10	0	8	8.7	9.5	1.2	7.2	2	6.2	8.5	7.2	9.2	8.5	8.7	8.2	7	9.7	7.5	5.2	1	10	8.2		5.1	9.3	5.8	6.3	6.7	6.3	5.6	5.8
37	*N. tenerifensis* DSM 44704[T]	3.5	5.5	2.7	2.2	3.2	3.5	6.5	6.7	3.2	2.2	4.7	5.7	5.5	6.2	4.5	4.2	4.7	6.7	4.7	4.2	5.2	5.2	5.2	4.2	6	5.5	5	4.7	3.5	6.7	4.2	3.5	4.5	6.7	4.2	4.5		8.5	4.6	4.8	7.2	6.5	5.8	5.5
38	*N. testacea* DSM 44765[T]	6.5	7.7	6.2	6	5.7	7.5	9	7.7	6.7	7.2	5.7	2.7	7.7	9	9.2	7	7.7	3.7	9.5	7	8.5	9.7	7.2	7.2	8.7	7.7	6.7	5.7	6.5	8.2	7.5	7.2	9.2	9.2	6.7	9.2	5.7		5.6	7	5.8	6.9	7	7.9
39	*N. transvalensis* DSM 43405[T]	7	7.5	7.5	8.5	7.7	9	4.7	6.7	8.2	9.7	9.2	11	8	10.5	11	7.7	8.7	11.2	12	7.5	10.2	9.7	5	9.2	10.2	7	8.7	9	7.2	5	8.2	9.5	11.7	10	7	11	8.7	11		5.1	4.6	3.2	4.9	6
40	*N. uniformis* JCM 3224[T]	6.2	9	5.7	7.5	5.7	7.5	8.7	5.7	8.2	8.5	6.5	1.5	9	9.5	9.5	7	7.7	0	9.7	6.7	8.7	9.2	7	7.2	9.2	9	7.5	6.2	6.2	8.5	8	7.2	9.5	9.5	6.7	9.5	6.7	3.7	11.2		4.6	4.6	5.3	5.6
41	*N. vaccinii* ATCC 11092[T]	5.7	8.5	5.7	7.2	6.2	7.7	5.7	5.2	8	9.5	8.2	9.7	8.5	10.2	10.7	6.2	7.5	9.5	11.2	6.7	10.5	9.5	5.7	8.2	9	8.5	7.2	8.5	5.7	5.7	7.2	7.7	11	9.2	5.7	10.7	8	9.7	6.7	9.5		2.6	3.7	4.9
42	*N. veterana* DSM 44445[T]	3.7	0.7	3.2	3.7	3.7	6.5	5.7	4.2	4.2	6.2	5.2	8.2	1.2	7.7	8.2	5.7	4.2	8.5	9.2	4.7	7.7	7.7	5.5	5.2	7.5	0.7	5.5	5.5	4	5.7	6.2	6	9	7.7	5.2	8.2	5	7.7	7	8.5	7.7		3.9	4.8
43	*N. vinacea* JCM 10988[T]	4.2	7	3.7	6.2	3.5	3.7	6.5	6.5	6.7	5.2	6	8.5	7	6.7	8.2	4.2	6.5	8.7	8	5	7	7.2	5.7	3.5	6.7	7	6.5	6.5	4.5	7.5	0.7	3.5	7.7	7	4.5	8.2	5	8.2	9	8.7	8	7		6.7
44	*N. yamanashiensis* DSM 44669[T]	5.2	8.2	6	7	6.2	5.2	7.7	8	7	5.7	7.5	8.5	8.2	6.2	7.2	6.5	7	8.7	7.5	6.2	8	1.5	6.2	6.7	5	8.2	6.7	7.5	6.7	7	5.5	4.7	7.2	5.7	6.5	7.2	5.2	9	9.2	8.7	8.5	7.7	6.2	

[a] Strain numbers were assigned arbitrarily.

[b] Values above the diagonal line indicate divergence in the 16S rRNA gene, and values below the diagonal line indicate divergence in the *hsp65* gene.

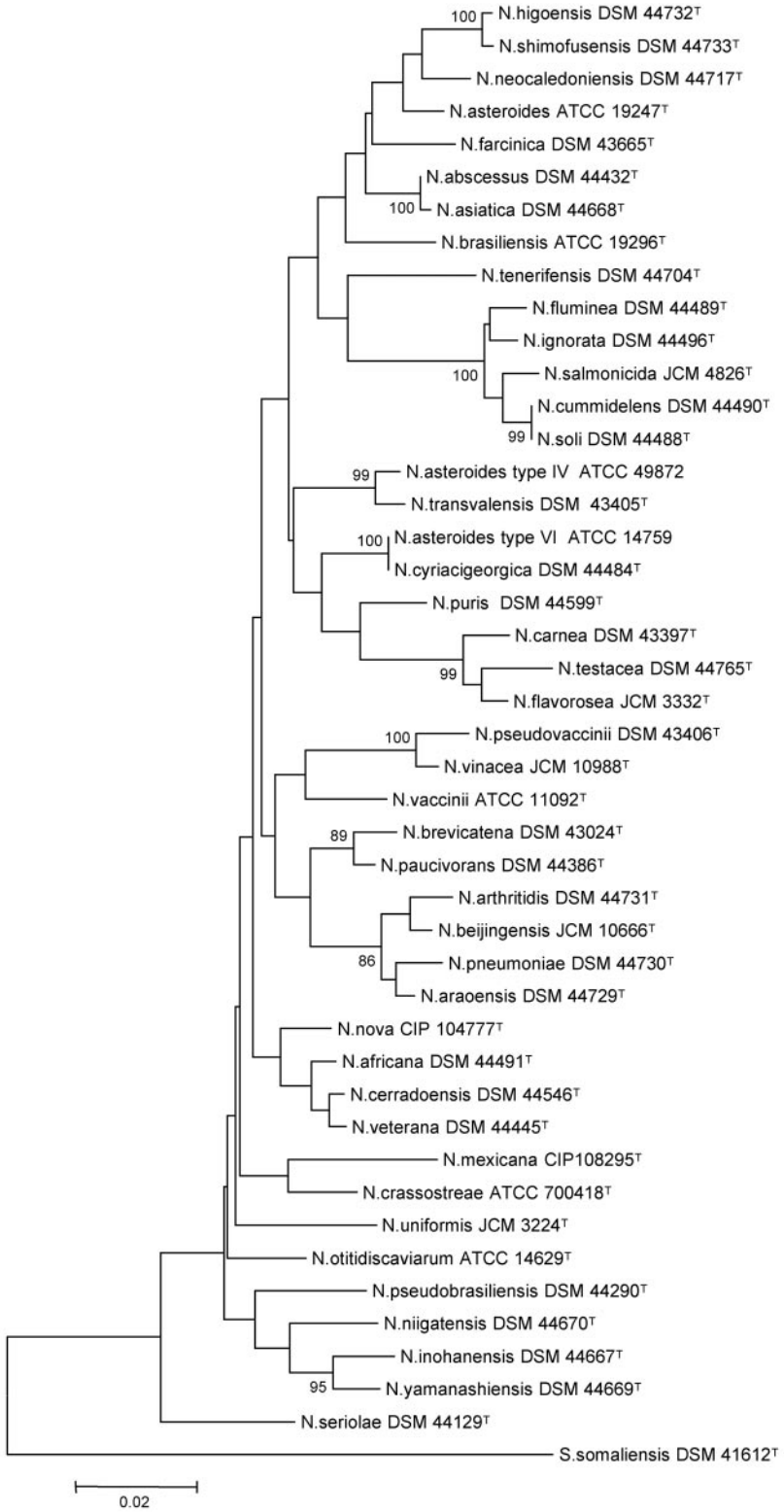


FIG. 2. Phylogenetic tree based on 16S rRNA sequencing of collection strains belonging to the genus *Nocardia*. The tree was constructed by using the neighbor-joining method, based on a 569-nucleotide stretch. Bootstrap values are expressed as a percentage of 1,000 replications. The scale bar represents 0.02 substitutions per nucleotide position.

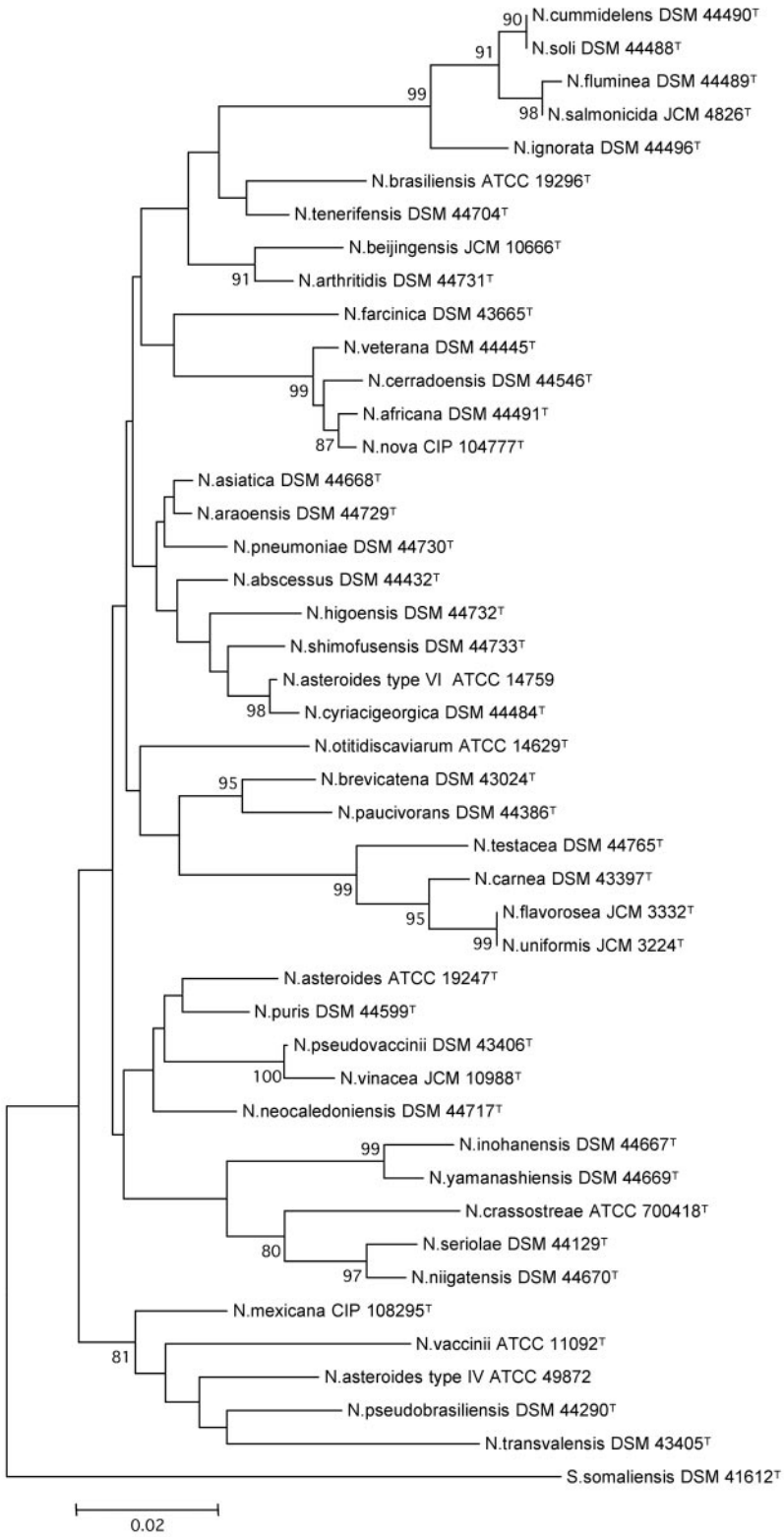


FIG. 3. Phylogenetic tree based upon *hsp65* sequencing of collection strains belonging to the genus *Nocardia*. The tree was constructed by using the neighbor-joining method, based on a 401-nucleotide stretch. Bootstrap values are expressed as a percentage of 1,000 replications. The scale bar represents 0.02 substitutions per nucleotide position.

TABLE 3. List of the three closest species obtained by using Bibi software applied to the 16S and *hsp65* databases for 21 clinical *Nocardia* isolates

Isolate	16S		*hsp65*	
	Species	% Dissimilarity	Species	% Dissimilarity
02.51	*N. farcinica*	0.0	*N. farcinica*	0.5
	N. higoensis	1.4	*N. aroensis*	3.2
	N. shimofusensis	1.5	*N. asiatica*	3.7
04.14	*N. farcinica*	0.3	*N. farcinica*	0.5
	N. shimofusensis	1.5	*N. aroensis*	3.2
	N. higoensis	1.7	*N. asiatica*	3.7
02.108	*N. abscessus*	0.9	*N. abscessus*	0.0
	N. asiatica	1.0	*N. aroensis*	0.9
	N. higoensis	2.7	*N. asiatica*	1.4
04.25	*N. abscessus*	0	*N. abscessus*	0.2
	N. asiatica	0.2	*N. aroensis*	1.1
	N. higoensis	1.9	*N. asiatica*	1.1
02.94	*N. abscessus*	0.3	*N. abscessus*	0.0
	N. asiatica	0.3	*N. aroensis*	0.9
	N. higoensis	2.2	*N. asiatica*	1.4
02.56	*N. brasiliensis*	0.5	*N. brasiliensis*	1.1
	N. abscessus	2.7	*N. tenerifensis*	2.5
	N. cyriacigeorgica	3.2	*N. asiatica*	3.9
04.21	*N. brasiliensis*	1.0	*N. brasiliensis*	1.1
	N. abscessus	3.2	*N. tenerifensis*	2.5
	N. farcinica	3.2	*N. asiatica*	3.9
00.69	*N. ignorata*	0.5	*N. ignorata*	0.2
	N. cummidelens	1.4	*N. cummidelens*	2.1
	N. soli	1.4	*N. soli*	2.1
03.14	*N. ignorata*	0.2	*N. ignorata*	0.0
	N. cummidelens	1.0	*N. cummidelens*	1.8
	N. soli	1.0	*N. soli*	1.8
N21	*N. ignorata*	0.2	*N. ignorata*	0.5
	N. cummidelens	1.0	*N. cummidelens*	2.1
	N. soli	1.0	*N. soli*	2.1
27.3837	*N. ignorata*	0.2	*N. ignorata*	0.0
	N. cummidelens	1.0	*N. cummidelens*	1.8
	N. soli	1.0	*N. soli*	1.8
00.18	*N. ignorata*	0.2	*N. ignorata*	0.2
	N. cummidelens	1.0	*N. cummidelens*	1.8
	N. soli	1.0	*N. soli*	1.8
04.12	*N. cyriacigeorgica*	0.0	*N. cyriacigeorgica*	1.4
	N. farcinica	2.6	*N. shimofusensis*	2.3
	N. abscessus	2.7	*N. pneumoniae* and *N. asiatica*	2.8
02.61	*N. cyriacigeorgica*	0.0	*N. cyriacigeorgica*	1.1
	N. farcinica	2.6	*N. shimofusensis*	2.1
	N. abscessus	2.7	*N. aroensis* and *N. asiatica*	2.5
02.112	*N. cyriacigeorgica*	0.0	*N. cyriacigeorgica*	1.4
	N. farcinica	2.6	*N. shimofusensis*	2.3
	N. abscessus	2.7	*N. pneumoniae* and *N. aroensis*	2.8
04.18	*N. nova*	0.0	*N. nova* and *N. africana*	0.7
	N. veterana	1.5		
	N. africana/cerradoensis	1.7	*N. veterana*	0.9
04.17	*N. nova*	0.2	*N. nova* and *N. africana*	0.9
	N. veterana	1.4		
	N. cerradoensis and N. africana	1.5	*N. veterana*	1.1
04.07	*N. cerradoensis*	0.2	*N. cerradoensis*	0.5
	N. veterana	0.3	*N. nova*	0.9
	N. africana	0.9	*N. africana*	0.9
02.11	*N. veterana*	0.3	*N. veterana*	0.0
	N. africana	0.9	*N. africana*	0.7
	N. nova	1.9	*N. nova*	0.7
02.132	*N. veterana*	0.3	*N. veterana*	0.0
	N. cerradoensis	0.9	*N. africana*	0.7
	N. africana	0.9	*N. nova*	0.7
02.44	*N. veterana*	0.2	*N. veterana*	0.0
	N. cerradoensis	0.7	*N. africana*	0.7
	N. africana	0.7	*N. nova*	0.7

ilarity) and *N. veterana* did not appear among the three closest species.

As it was the first description of clinical isolates belonging to *N. cerradoensis* and *N. ignorata* (03.14, N21, 27.3837, 00.18, and 04.07), we have confirmed species identification by an extensive phenotypic and chemotaxonomic analysis for all isolates (data not shown).

DISCUSSION

In the mid-1990s, Wallace et al. (33), Steingrube et al. (28, 29), and Laurent et al. (15) described new approaches to *Nocardia* genus and species identification. Here, when we included the restriction profiles of the 36 new *Nocardia* species in the decision tree described in 1997 by Steingrube et al., a significant number of species had identical profiles (Fig. 1). Thus, the "*N. asteroides* type VI" pattern (corresponding to drug susceptibility types) (34) according to the work of Steingrube et al. (28, 29) was identical to the pattern obtained for *N. abscessus*, *N. asteroides* (ATCC 19247^T), *N. brevicatena*, *N. cyriacigeorgica*, *N. paucivorans*, and *N. vinacea.* This pattern is also obtained for 60% of French clinical isolates (F. Laurent, personal data). On the other hand, it was not possible to distinguish *N. africana*, *N. cummidelens*, *N. nova*, *N. soli*, *and N. veterana*. These results confirmed that PRA is unsuitable for delineating the new nocardial taxonomic background (8, 22). Moreover, we found that the PRA pattern for *N. abscessus*, previously included in the *N. asteroides* type I subspecies, differed from the profile reported by Steingrube (Fig. 1). This was confirmed according to 21 clinical isolates of *N. abscessus*/*N. asiatica* (unpublished data).

Thus, the use of PRA can lead to erroneous species identification of both clinical and environmental *Nocardia* isolates. Pottumarthy et al. (22) recently reported that the *N. veterana hsp65* PRA profile was identical to that of *N. nova* (22). The absence of the newly described *Nocardia* species in the PRA tree inevitably leads to an overestimation of the prevalence of infections due to the species included in the tree and to an underestimation of infections due to species that are missing from the tree. For instance, in our large collection of clinical strains, for which *hsp65* PRA was used for species identification, a recent reexamination based on 16S sequence analysis demonstrated that 34% and 26% of strains with an *N. asteroides* type VI PRA pattern in fact belonged to the species *N. cyriacigeorgica* and *N. abscessus*, respectively (the other isolates belonged to *N. transvalensis*, *N. paucivorans*, *N. brevicatena*, or *N. asteroides* or were identified as *Nocardia* sp.) (F. Laurent, personal data).

BstEII enzyme restriction provides rapid and simple separation of *Mycobacterium* with PRA (28, 29). Other actinomycetes, including *Nocardia*, were reported to have no BstEII restriction site. However, we found that the *Nocardia* species *ignorata* and *cerradoensis* had a BstEII restriction site. Moreover, Brunello et al. (3) showed that several *Mycobacterium* species (*M. confluentis*, *M. gilvum*, *M. tusciae*, *M. brumae*, *M. pulveris*, *M. duvalii*, *M. szulgai*, and *M. gadium*) had no BstEII restriction site and had a BstEII restriction profile identical to that of *Nocardia* strains. This underlines the importance of the *Nocardia*-specific 16S rRNA PCR method (15), which is the only approach currently capable of offering rapid and accurate identification of the genus *Nocardia* (15).

hsp65 PRA has been shown to be unreliable for identification of *Nocardia* species before (8, 22). Our results confirm and extend these findings. The use of new restriction enzymes together with a new dichotomous decision tree failed to improve *Nocardia* species identification, and the inseparable clusters included the most common clinical species. PRA explores only the polymorphism of a few bases present at the restriction site of each enzyme, and it would be difficult to differentiate more than 40 species on the basis of such a small region of an amplified fragment.

Advances in DNA sequencing and the increasing number of sequences available in databases have greatly improved the molecular identification of various bacteria. For *Nocardia*, tools such as the Ribosomal Differentiation of Medical Microorganisms (RIDOM) database (19) and the MicroSeq kit (Perkin-Elmer Applied Biosystems) (7) are based on the 16S rRNA gene. The latter authors highlighted the value of such an approach but stressed that (i) interspecies heterogeneity of closely related nocardial species is very low, (ii) results for a given isolate are not always congruent and conclusive when using different databases of a given gene (7, 19, 25), and (iii) the most important component for successful identification of bacterial isolates is an accurate and complete database (7). But at present, validated databases for the molecular identification of *Nocardia* isolates are available with a commercial kit (MicroSeq; Applied Biosystems, Foster City, Calif.) or are not freely accessible (RIDOM). Moreover, these databases comprise fewer than 30 validated *Nocardia* species. This is why we decided to develop specific databases of *hsp65* and 16S rRNA sequences for all validated and putative *Nocardia* species (as of 31 October 2004, except for *N. alba*, *N. pigrifrangens*, and *N. caishijiensis*) and compared their performance.

The sequenced 16S rRNA region corresponds to bases 64 to 663 of the *E. coli* 16S rRNA gene. It is very close to the region used in the RIDOM database (bases 54 to 510) and the MicroSeq 500 database (bases 4 to 532) and includes the most variable regions of the nocardial 16S rRNA gene. We observed a higher average dissimilarity among the different species with the *hsp65* sequence (mean, 6.3%; range, 12 to 0%) than with the 16S rRNA sequence (mean, 5.2%; range, 9.5 to 0%). In addition, the distances between each pair of species were three to four times larger with *hsp65* than with 16S rRNA. Likewise, the number of variable sites was larger in the *hsp65* gene sequence (26.4% versus 16.5%). Whereas variable sites are confined to certain areas of the 16S rRNA gene, they are dispersed throughout the *hsp65* gene and show a higher frequency in two regions (positions 624 to 664 and positions 683 to 725), as previously reported for mycobacteria by Ringuet et al. (23). Most substitutions in the *hsp65* gene are confined to the third position of each codon (the wobble position), ensuring that the resulting amino acid sequence is preserved across the genus (data not shown), together with the crucial function of Hsp65 protein in resistance to environmental stress (23).

The relative positions of each species were similar in the *hsp65* and 16S rRNA phylogenetic trees when bootstrap values higher than 80% were examined. However, the tree based on *hsp65* was more discriminatory and more robust. Respectively, 23 nodes (55%) and 17 nodes (41%) were supported by boot-

strap values greater than 50% and 80% in the *hsp65* tree, compared to only 21 (50%) and 11 (26%) nodes in the 16S rRNA tree.

Some species that were not clearly distinguished by their 16S rRNA sequences were clearly distinguished by their *hsp65* sequences; this was the case for *N. shimofusensis* versus *N. higoensis* and *N. abscessus* versus *N. asiatica*, for example. In contrast, *N. flavorosea* and *N. uniformis* had identical *hsp65* sequences but very different 16S rRNA sequences. *N. soli* and *N. cummidelens* could not be separated by using either database. For *N. cyriacigeorgica* and *N. asteroides* type VI (ATCC 14759), 16S rRNA sequences and *hsp65* sequences revealed 0% and 0.5% dissimilarity, respectively. These data reinforced the idea that these two species form in fact a single taxon as recently suggested by Roth et al. and Patel et al. (21, 25).

Analysis of the clinical isolates indicated that (i) species discrimination was better with *hsp65* than with 16S rRNA (larger distances between the closest species pairs) and (ii) combined analysis of the two databases resolved some inconclusive results obtained with only one database. How "similar" or "close" a strain must be to the reference strain before it can be considered a species is controversial, as is the choice between similarity (or dissimilarity) and phylogenetic analysis for species designation. Some authors answer this question by statistical analysis. For instance, Cloud et al. used a reporting criterion of ≥99% similarity (or ≤1% dissimilarity) and a statistical error probability of 3% to separate two species with the MicroSeq 500 kit, according to the normal distribution of pairwise distances (7). Meanwhile, Mellmann et al. chose a reporting criterion of ≥99.12% similarity (or ≤0.88% dissimilarity) to define a distinct species and calculated an error probability of 1% with the RIDOM database (19). Whatever the criterion and the database, the choice is arbitrary and will likely change as new species are added to the database. It is interesting that, according to the chosen statistical approach, it is possible to define, from a threshold, a residual error to attribute a strain to a given species although it does not belong to this species. However, in the absence of data on intraspecies variability, Mellmann et al. and Cloud et al. cannot quantify the probability of not rightfully attributing a strain to a given species. In a recent review (5), Clarridge stated that no similarity or dissimilarity value could be assigned to a defined species on the basis of its 16S rRNA sequence. This is partly because different values are generated by the use of different databases and different methods (5) and emphasizes the need for clean, updated, and controlled reference databases.

Nocardia taxonomic studies must allow new bona fide species to be identified on the basis of morphological, biochemical, physiological, and chemotaxonomic properties coupled with genomic and phylogenetic analyses (27), while species identification of clinical isolates must be based on conformity to the phylogenically closest known species contained in updated and comprehensive databases. The latter task is greatly facilitated by new bioinformatics tools based on simplified analysis of sequence similarities (or dissimilarities) within an accepted and updated bacterial framework. One such tool, named Bibi, was recently described by Devulder et al. (9), but it requires complete and accurate sequence data on relevant genes if it is to be used for *Nocardia* identification.

The choice of genes is indeed crucial. According to the work of Goebel and Stackebrandt (11), 16S rRNA dissimilarity of ≥3% always clearly separates isolates belonging to different species; in other words, a strain does not belong to a given species if its 16S rRNA sequence differs by more than 3%. However, some species of *Nocardia* have identical 16S sequences (Fig. 2), as is the case for many other taxa. As expected for a protein-encoding gene, the *Nocardia hsp65* gene is less conserved than the 16S rRNA gene.

In conclusion, our results suggest that *hsp65* sequencing has the potential for providing a reliable means of identifying clinical *Nocardia* isolates to the genus and species level. The *hsp65* sequences presented here can be used to establish a new database, analogous to the 16S rRNA gene database, containing type strain sequences that serve as standards for identification. In addition, combining the *hsp65* and 16S rRNA sequences could form the basis for a new *Nocardia* species identification system, particularly for species with similar 16S rRNA sequences. A multilocus sequencing strategy, including a specific, clean, and controlled database containing all validated type strains belonging to the genus *Nocardia* and to closely related genera (*Mycobacterium*, *Rhodococcus*, *Tsukamurella*, *Gordonia*, etc.), would answer the new taxonomic background of the actinomycetes family.

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